

[see, Weiland, et al., Blut, 44, 173-175 (1982)] or utility in other contexts, such as in assays of hpG-CSF antagonism. Competitive antagonists may be quite useful in, for example, cases of overproduction of hpG-CSF.

II. REMARKS

AMENDMENTS TO THE SPECIFICATION

By the foregoing amendment, the applicant has updated the claim of priority on page 1 of the specification and introduced amendments previously made in parent applications, to correct obvious typographical errors. No new matter has been added. This "Preliminary Amendment" is accompanied by a "Supplemental Information Disclosure Statement" and accompanying Form PTO-1449, and "Response to 'Notice to Comply with Requirements for Patent Applications Containing Nucleotide Sequence and/or Amino Acid Sequence Disclosure'."

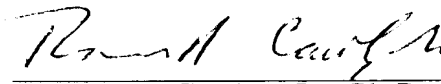
III. CONCLUSION

In view of the foregoing, the applicant respectfully submits that the proposed amendments may properly be entered and respectfully requests that substantive examination be undertaken.

Respectfully submitted,

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March 6, 2002

EXHIBIT A
VERSION WITH MARKINGS TO SHOW CHANGES MADE

Please replace the paragraph beginning on page 1, line 29 with the following rewritten paragraph:

--It is believed that small amounts of certain hematopoietic growth factors account for the differentiation of a small number of progenitor "stem cells" into the variety of blood cell lines, for the tremendous proliferation of those lines, and for the ultimate differentiation of mature blood cells from those lines. Because the hematopoietic growth factors are present in extremely small amounts, the detection and identification of these factors has relied upon an array of assays which as yet only distinguish among the different factors on the basis of stimulative effects on cultured cells under artificial conditions. As a result, a large number of names have been coined to denote a much smaller number of factors. As an example of the resultant confusion the terms, IL-3, BPA, multi-CSF, HCGF, MCGF and PSF are all acronyms which are now believed to apply to a single murine hematopoietic growth factor. Metcalf, Science, 229,:16-22 (1985). See also, Burgess, et al. J.Biol.Chem., 252, ~~1988~~ 1998-2003 (1977), Das, et al. Blood, 58, ~~600~~ 630-641 (1981), Ihle, et al., J. Immunol., 129, 2341 (1982), Nicola, et al., J. Biol.Chem., 258,9017 (1983), Metcalf, et al., Int.J.Cancer, 30,773 (1982), and Burgess, et al. Int.J.Cancer, 26, 647 (1980), relating to various murine growth regulatory glycoproteins.

Please replace the paragraph beginning on page 5, line 35 with the following rewritten paragraph:

Novel DNA sequences of the invention include sequences useful in securing expression in procaryotic or eucaryotic host cells of polypeptide products having at least a part of the primary structural conformation and one or more of the biological properties of naturally occurring pluripotent granulocyte colony-stimulating factor. DNA sequences of the invention are specifically seen to comprise: (a) the DNA sequence set forth in ~~Table VH and Table VII~~ FIG. 2 and FIG. 3 or their ~~complimentary~~ complementary strands; (b) a DNA sequence which hybridizes (under hybridization conditions such as illustrated herein or more stringent conditions) to the DNA sequences in ~~Table VH~~ FIG. 2 or to fragments thereof; and (c) a DNA sequence which, but for the degeneracy of the genetic code, would hybridize to the DNA sequence in ~~Table VH~~ FIG. 2. Specifically comprehended in part (b) are genomic DNA sequences encoding allelic variant forms of hpG-CSF and/or encoding other mammalian species of pluripotent granulocyte colony-stimulating factor. Specifically

comprehended by part (c) are manufactured DNA sequences encoding hpG-CSF, fragments of hpG-CSF and analogs of hpG-CSF which DNA sequences may incorporate codons facilitating translation messenger RNA in microbial hosts. Such manufactured sequences may readily be constructed according to the methods of Alton, et al., PCT published application WO 83/04053.

Please replace the paragraph beginning on page 6, line 25 with the following rewritten paragraph:

Also comprehended by the present invention is that class of polypeptides coded for by portions of the DNA complement to the top strand human cDNA or genomic DNA sequences of ~~Tables VII or VIII~~ FIGS. 2 or 3 herein, i.e., "complementary inverted proteins" as described by Tramontano, et al., Nucleic Acids Res., 12, 5049-5059 (1984).

Please replace the paragraph beginning on page 8, line 27 with the following rewritten paragraph:

~~The Figure~~ FIG. 1 is a partial restriction endonuclease map of the hpG-CSF gene accompanied by arrows depicting the sequencing strategy used to obtain the genomic sequence. Figures 2 through 10 are DNA sequences according to the invention.

Please replace the paragraph beginning on page 11, line 28 with the following rewritten paragraph:

In order to obtain a sufficient amount of pure material to perform suitably definitive amino acid sequence analysis, cells of a bladder carcinoma cell line 5637 (subclone1A6) as produced at Sloan-Kettering were obtained from Dr. E. Platzer. Cells were initially cultured in Iscove's medium (GIBCO, Grand Island, New York) in flasks to confluence. When confluent, the cultures were trypsinized and seeded into roller bottles (1-1/2 flasks/bottle) each containing 25 ml of preconditioned Iscove's medium under 5% CO₂. The cells were grown overnight at 37°C. at 0.3 rpm.

Please replace the paragraph beginning on page 13, line 25 with the following rewritten paragraph:

Samples, ranging in volume from 126 ml to 850 ml and containing 1-8 mg of protein, about 10% of which was hpG-CSF, were loaded onto the column at a flow rate ranging from 1 ml to 4 ml per minute. After loading and an initial washing with 0.1M ammonium acetate (pH 6.0-7.0) in 80% 2-propanol at a flow rate of ~~1/ml/min.~~ One 1/ml/min, one milliliter fractions were collected and monitored for proteins at 220nm, 260nm and 280nm.

Please replace the paragraph beginning on page 13, line 33 with the following rewritten paragraph:

As a result of purification, fractions containing hpG-CSF were clearly separated (as fractions 72 and 73 of 80) from other protein-containing fractions. ~~HpG-CSF~~ hpG-CSF was isolated (150-300 μ g) at a purity of about $85 \pm 5\%$ and at a yield of about 50%. From this purified material 9 μ g was used in Run #4, an amino acid sequence analysis wherein the protein sample was applied to a TFA-activated glass fiber disc without polybrene. Sequence analysis was carried out with an AB 470A sequencer according to the methods of Hewick, et al., J.Biol. Chem., **256**, 7990-7997 (1981) and Lai, Anal. Chim. Acta, **163**, 243-248 (1984). The results of Run #4 appear in Table III.

Please replace the paragraph beginning on page 15, line 16 with the following rewritten paragraph:

The amino acid sequence ~~give~~ given in Table IV was sufficiently long (44 residues) and unambiguous to construct probes for obtaining hpG-CSF cDNA as described infra.

Please replace the paragraph beginning on page 16, line 9 with the following rewritten paragraph:

Total RNA was extracted from approximately 1 gram of cells from a bladder carcinoma cell line 5637 (1A6) using a guanidinium ~~thiocynate~~ thiocyanate procedure for quantitative isolation of intact RNA. [Chirgwin, et al., Biochemistry, **18**, 5294-5299 (1979)].

Please replace the paragraph beginning on page 17, line 4 with the following rewritten paragraph:

Hybridization probes designed on the basis of the hpG-CSF amino terminal sequence of Table IV consisted of a set of 24 oligonucleotides each being 23 bases in length and containing three inosine residues. The probe oligonucleotides were manufactured according to the procedure of Caruthers, et al., Genetic Engineering, **4**, 1-18- 1-17 (1982) and labeled with γ - 32 P ATP by kinasing with polynucleotide kinase. The probe oligonucleotides, corresponding to the messenger RNA for residues 23-30 of the sequence of Table IV, are illustrated in Table V.

Please replace the paragraph beginning on page 19, line 10 with the following rewritten paragraph:

The filter representing N-myc probe 3 gave a very weak signal relative to the other four probed filters and was not washed any further. After a 10 minute 50°C. wash, the

Geiger counter gave the following percent signal with probe one being normalized to 100%: Probe 2, 20%; Probe 3 (~~45%~~) (45), 2%; Probe 4, 92%; and Probe 5, 75%. After a 55°C. wash, the percentages were: Probe 2, 16%; Probe 4, 100%; and Probe 5, 80%. A final wash at 60°C. yielded the following percentages: Probe 2, 1.6%; Probe 4, 90%; and Probe 5, 70%.

Please replace the paragraph beginning on page 21, line 19 with the following rewritten paragraph:

Sequencing of the recombinant hpG-CSF cDNA clone, Ppo2, obtained by the above procedure was accomplished by the dideoxy method of Sanger, et al., Proc. Natl. Acad. Sci. (USA) 74, 5463-5467 (1977). The single-stranded DNA phage M-13 was used as a cloning vector for supplying single-stranded DNA templates from the double-stranded cDNA clones. The Sanger, et al., method revealed the sequence as set forth in ~~Table VII~~ FIG. 2 accompanied by its amino acid translation and a complementary strand in the polypeptide coding region.

Please delete the entire text of pages 22 through 24, inclusive (i.e., Table VII).

Please replace the paragraph beginning on page 25, line 1 with the following rewritten paragraph:

The following characteristics of the sequence of ~~Table VII~~ FIG. 2 are of note. At the 5' end of the sequence there are shown bases corresponding to those of the poly G cDNA linker. There then occur about five bases (designated as "N") whose sequence could not readily be determined unambiguously by the Sanger, et al. method due to the preceding multiple G's. The sequence thereafter reveals a series of 12 codons encoding a portion of a putative leader sequence for the polypeptide. Based on correspondence to the amino terminal sequence of natural isolates of hpCSF described in Example 1, the initial threonine residue of the putative "mature" form of hpG-CSF is indicated by +1. Mature hpG-CSF is thereafter revealed to include 174 residues as indicated. Following the "stop" codon (the OP codon, TGA) are approximately 856 bases of an untranslated 3' sequence and multiple A's of the poly A "tail". Unique HgiAi, and ApaI restriction endonuclease recognition sites, as well as two StuI sites (discussed infra with respect to construction of procaryotic and eucaryotic expression systems) are also designated in ~~Table VII~~ FIG. 2. Owing to the lack of asparagine residues in the polypeptide, there are no apparent sites for N-glycosylation. The underscored 6 bases near the end of the 3' untranslated sequence represent a potential polyadenylation site.

Please replace the paragraph beginning on page 26, line 19 with the following rewritten paragraph:

In this example, cDNA encoding hpG-CSF as isolated in the previous example was used to screen a genomic clone. A phage lambda human fetal liver genomic library [prepared according to the procedure of Lawn, et al. Cell, 15, 1157-1174 (1978) and obtained from T. Maniatis] was screened using a nick translated probe consisting of two hpG-CSF cDNA fragments isolated by digestion with HgiAI and StuI (HgiAI to StuI, 649 b.p.; StuI to StuI, 639 b.p.). A total of approximately 500,000 phage were plated on 12 (15 cm) petri dishes and plaque lifted and hybridized to probe using the Benton/Davison procedure [Benton, et al., Science, 196, 180 (1977)]. A total of 12 positive clones were observed. Three clones (1-3) yielding the strongest signals upon autoradiography in a secondary screening were grown in 1 liter cultures and mapped by restriction enzyme digestion and Southern blotting using a radio-labeled 24-mer oligonucleotide (kinased with γ -³²P ATP) 5' CTGCACTGTCCAGAGTGCAGTGTG3'. The mapping results showed that isolates 1 and 3 were identical and 2 contained 2000 additional bases 5' to the hpG-CSF gene. Therefore, clone 2 was used for further characterization. DNA from clone 2 was digested with R1 to release an 8500 bp hpG-CSF containing fragment which was subsequently subcloned into pBR322 and further mapped by restriction endonuclease digests, Southern Blotting, M13 subcloning and sequencing. The sequence obtained is as set out in ~~Table VIII~~ FIG. 3.

Please delete the entire text of pages 28 through 32, inclusive (i.e., Table VIII).

Please replace the paragraph beginning on page 33, line 1 with the following rewritten paragraph:

A restriction endonuclease map (approximately 3.4 Kb) of genomic DNA containing the hpG-CSF gene is detailed in Figure 1. The restriction endonucleases shown in Figure 1 are: NcoI, N; PstI, P; BamHI, B; ApaI, A; XhoI, X; and KpnI, K. The arrows below the map depict the sequencing strategy used to obtain the genomic sequence. The boxed regions are those found in the cDNA clone with the dashed open ended box representing sequence not present in the cDNA clone, but identified by probing mRNA blots. The identification of coding sequences proposed for ~~exon one~~ exon 1 was carried out by Northern blot analysis. A 24 mer oligonucleotide probe 5' CAGCAGCTGCAGGGCCATCAGCTT3', spanning the predicted splice junctures for exons 1 and 2 was hybridized to hpG-CSF mRNA in a Northern blot format. The resulting blot shows an mRNA the same size (~ 1650 bp) as that seen with an exon 2 oligonucleotide probe. This data combined with the ability to direct expression of hpG-CSF from the

pSVGM-Ppol vector (Example 9) using the Met initiation codon depicted in ~~Table VII~~ FIG. 3, defines the coding sequences contained in exon 1. Exons 2-5 are defined by the coding sequences obtained in the cDNA clone (Ppo2) of the hpG-CSF gene (~~Table VII~~) (FIG. 2).

Please replace the paragraph beginning on page 34, line 5 with the following rewritten paragraph:

The construction of ~~Sections I, II and III~~ Sections I, II and III is illustrated in ~~Table IX through XIV~~ Tables VII through IX and FIGS. 4-6. In the construction of Section I, as illustrated in ~~Tables IX and X~~ Table VII and FIG. 4, oligonucleotides 1-14 were assembled into 7 duplexes (1 and 8); 2 and 9; 3 and 10; 4 and 11; 5 and 12; 6 and 13; and 7 and 14). The 7 duplexes were then ligated to form Section I as shown in ~~Table X~~ FIG. 4. It may also be noted in ~~Table X~~ FIG. 4, that Section I includes an upstream XbaI sticky end and a downstream BamHI sticky end useful for ligation to amplification and expression vectors and for ligation to Section II.

Page 35, line 1, please replace "Table IX" with --Table VII--.

Please delete the entire text of page 36 (i.e., Table X).

Please replace the paragraph beginning on page 37, line 1 with the following rewritten paragraph:

As illustrated in ~~Tables XI and XII~~ Table VIII and FIG. 5, in the construction of Section II, oligonucleotides 15-30 were assembled into 8 duplexes (15 and 23; 16 and 24; 17 and 25; 18 and 26; 19 and 27; 20 and 28; 21 and 29; and 22 and 30). These 8 duplexes were then ligated to form Section II, as shown in ~~Table XII~~ FIG. 5. As further shown in ~~Table XII~~ FIG. 5, Section II has an upstream BamHI sticky end and a downstream EcoRI sticky end useful for ligation to an amplification vector and for ligation to Section I. Near its downstream end, Section II also includes a downstream SstI site useful in the eventual ligation Sections II and III.

Page 37, line 14, please replace "Table XI" with --Table VIII--.

Please delete the entire text of page 38 (i.e., Table XII).

Please replace the paragraph beginning on page 39, line 1 with the following rewritten paragraph:

Finally, Section III was constructed as shown in ~~Tables XIII and XIV~~ Table IX and FIG. 6. For this construction, oligonucleotides 31-42 were assembled into 6 duplexes (31 and 37; 32 and 38; 33 and 39; 34 and 40; 35 and 41; and 36 and 42). The 6 duplexes were then ligated to form Section III as depicted in ~~Table XIV~~ FIG. 6. As also shown in ~~Table XIV~~ FIG. 6, Section III includes an upstream BamHI sticky end and a downstream EcoRI sticky end useful for ligating into an amplification vector, and at least in the case of the EcoRI end, into an expression vector. In addition, Section II has an upstream SstI site useful in the eventual ligation of Sections II and III.

Page 39, line 15, please replace "Table XIII" with --Table IX--.

Please delete the entire text on page 40 (i.e., Table XIV).

Please replace the paragraph beginning on page 41, line 10 with the following rewritten paragraph:

The vector containing Sections I and II is digested with XbaI and SstI. Likewise, the vector containing Section III is digested with SstI and EcoRI. Both of the smaller of the two fragments resulting from each digestion are ligated into ~~a~~ a plasmid pCFM1156 which is previously opened with XbaI and EcoRI. The product of this reaction is an expression plasmid containing a continuous DNA sequence, as shown in ~~Table XV~~ FIG. 7, encoding the entire hpG-CSF polypeptide with an amino terminal methionine codon (ATG) for E.coli translation initiation.

Please delete the entire text of pages 42-43 (i.e., Table XV).

Please replace the paragraph beginning on page 44, line 1 with the following rewritten paragraph:

Although any suitable vector may be employed to express this DNA, the expression plasmid pCFM1156 may readily be constructed from a plasmid pCFM836, the construction of which is described in published European Patent Application No. 136,490. pCFM836 is first cut with NdeI and then blunt-ended with PollI such that both existing NdeI sites are destroyed. Next, the vector is digested with ClaI and SacII to remove an existing polylinker before ligation to a substitute polylinker as illustrated in ~~Table XVI~~ FIG. 8. This substitute polylinker may be constructed according to the procedure of Alton, et al., supra. Control of expression in the expression pCFM1156 plasmid is by means of a lambda P_L promoter, which itself may be under the control of a C₁₈₅₇ repressor gene (such as is provided in E.coli strain K12 Δ Htrp).

Please delete the entire text of page 45 (i.e., Table XVI).

Please replace the paragraph beginning on page 46, line 8 with the following rewritten paragraph:

Plasmid Ppo2, containing the hpG-CSF gene shown in ~~Table VH~~ FIG. 2, was digested with HgiAI and StuI providing an approximately 645 base pair fragment including the gene for mature hpCSF (as shown in ~~Table VH~~ FIG. 2) with seven of the leader sequence residue codons at the 5' end and about 100 base pairs of the 3' non-coding region. HgiAI digestion leaves a 5', 4-base sticky end identical to that of PstI, and StuI leaves a blunt end. This allows for ready insertion of the fragment into M13 mp8 (Rf) cut with PstI and with the blunt-end-forming restriction enzyme, HincII. Upon amplification in M13, the hpG-CSF DNA was excised by digestion with ApaI and BamHI which cut, respectively, at the ApaI site spanning the codons for residues +3 to +5 of hpCSF and at a BamHI site "downstream" of the HincII site in the M13 mp8 restriction polylinker. In order to allow for E. coli expression of the hpG-CSF polypeptide, a synthetic fragment was prepared as set out in ~~Table XVH~~ Table X below.

Page 46, line 28, please replace "Table XVII" with --Table X--.

Please replace the paragraph beginning on page 47, line 1 with the following rewritten paragraph:

As may be determined from analysis of ~~Table XVH~~ Table X, the linker includes an ApaI sticky end, codons specifying the initial three residues of the amino terminal of hpG-CSF ("restoring" the Thr¹, Pro², Leu³-specifying codons deleted upon ApaI digestion of the M13 DNA described above and employing codons preferentially expressed in E. coli), a translation initiating ATG, a sequence of 24 base pairs providing a ribosome binding site, and an XbaI sticky end.

Please replace the paragraph beginning on page 47, line 30 with the following rewritten paragraph:

The level of expression of hpG-CSF by the transformed cells was estimated on a ~~SDS-poly acrylamide~~ SDS-polyacrylamide gel stained with coomassie blue dye to be 3-5% of total cellular protein.

Please replace the paragraph beginning on page 48, line 12 with the following rewritten paragraph:

A second purification procedure was developed to yield larger quantities of hpG-CSF formulated in a nonorganic-containing buffer. This material is suitable for in vivo studies. One hundred and fifty grams of cell paste was resuspended in about 600 ml of 1 mM DTT and passed 4 times through a Manton Gualin Homogenizer at about 7000 PSI. The broken cell suspension was centrifuged at 10,000 g for 30 minutes and the pellet was resuspended in 400 ml of 1 % deoxycholate (DOC), 5 mM EDTA, 5 mM DTT, and 50 mM Tris, pH 9. This suspension was mixed at room temperature for 30 minutes and centrifuged at 10,000 g for 30 minutes. The pellet was resuspended in about 400 ml of water and centrifuged at 10,000 g for 30 minutes. The pellet was solubilized in 100 ml of 2% Sarkosyl and 50 mM Tris at pH 8. CuSO_4 was added to 20 μM and the mixture was stirred 16 hours at room temperature, and then centrifuged at 20,000 g for 30 minutes. To the supernatant was added 300 ml acetone. This mixture was put on ice for 20 minutes and then centrifuged at 5000 g for 30 minutes. The pellet was dissolved in 250 ml of 6 M guanidine and 40 mM sodium acetate at pH 4, and put over a 1,200 ml G-25 column equilibrated and run in 20 mM sodium acetate at pH 5.4. The hpG-CSF peak (about 400 ml) was pooled and put on a 15 ml CM-cellulose column equilibrated in 20 mM sodium acetate at pH 5.4. After loading, the column was washed with 60 ml of 20 mM sodium acetate at pH 5.4 and with 25 mM sodium chloride, and then the column was eluted with 200 ml of 20 mM sodium acetate at pH 5.4 and with 37 mM sodium chloride. 150 ml of this eluent was concentrated to 10 ml and applied to a 300 ml G-75 column equilibrated and run in 20 mM sodium acetate and 100 mM sodium chloride at pH 5.4. The peak fractions comprising 35 ml were pooled and filter sterilized. The final concentration of hpG-CSF was 1.5 mg/ml, is was greater than 95% pure as determined by analysis on a gel, and contained less than 0.5 ng of pyrogen per 0.5 mg of hpG-CSF. The pyrogen level was determined using a Limulus Amebocyte Lysate (LAL) test kit (M. A. Bioproducts, Walkersville, Maryland).

Please replace the paragraph beginning on page 49, line 24 with the following rewritten paragraph:

Site directed mutagenesis procedures according to Souza, et al., published PCT Application No. WO85/00817, published February 28, 1985, were carried out on [Met¹] encoding DNA of plasmid p536P_{po2}, described infra, using synthetic oligonucleotides ranging in size from 20 to 23 bases as set out in ~~Table XVIII~~ Table XI below. Oligonucleotide No. 1 allowed for formation of a gene encoding [Ser¹⁷]hpG-CSF; oligonucleotide No. 2 allowed for formation of [Ser³⁶]hpG-CSF, and so on.

Page 50, line 1, please replace "Table XVIII" with --Table XI--.

Page 50, line 28, please replace "Table XIX" with --Table XII--.

Please replace the paragraph beginning on page 51, line 1 with the following rewritten paragraph:

In a purification procedure for Cys to Ser analogs according to the present invention, about 10-15 g of cell paste was resuspended in 40 ml of 1 mM DTT and passed 3 times through a French Pressure Cell at 10,000 psi. The broken cell suspension was centrifuged at 1,000 g for 30 minutes. The pellet was resuspended in 1% DOC, 5 mM EDTA, 5 mM DTT, 50 mM Tris, pH 9 and allowed to mix 30 minutes at room temperature. The mixtures was centrifuged at 10,000 g for 30 minutes, resuspended in 40 ml H₂O, and recentrifuged ~~as~~ at 10,000 g for 30 minutes. The pellet was dissolved in 10 ml of 2% Sarkosyl, 50 mM DTT, 50 mM Tris, pH 8. After mixing for 1 hour, the mixture was clarified by centrifugation at 20,000 g for 30 minutes, and then applied to a 300 ml G-75 column equilibrated and run in 1% Sarkosyl, 50 mM Tris, pH 8. Fractions containing the analog were pooled and allowed to air oxidize by standing with exposure to air for at least one day. Final concentrations ranged from 0.5 - 5 mg/ml.

Please replace the paragraph beginning on page 51, line 24 with the following rewritten paragraph:

In this example, a mammalian cell expression system was devised to ascertain whether an active polypeptide product of hpG-CSF DNA could be expressed in and secreted by mammalian cells (COS-1, A.T.C.C. CRL-1650). This system was designed to provide for secretion of a polypeptide analog of ~~hpGCSF~~ hpG-CSF via expression and secretory processing of a partially synthetic, partially cDNA-derived construction encoding [Ala¹] hpG-CSF preceded by a leader polypeptide having the sequence of residues attributed to human GM-CSF in Wong, et al., Science, 228, 810-815 (1985) and Lee, et al., Proc. Natl. Acad. Sci. (USA), 82, 4360-4364 (1985).

Please replace the paragraph beginning on page 52, line 13 with the following rewritten paragraph:

The specific manipulations involved in the expression vector construction were as follows. A leader-encoding DNA sequence was synthesized as set out in ~~Table XX~~ Table XIII below.

Page 52, line 20, please replace "Table XX" with --Table XIII--.

Please replace the paragraph beginning on page 53, line 1 with the following rewritten paragraph:

As indicated in ~~Table XX~~ Table XIII, the sequence includes HindIII and Apal sticky ends and codons for the 17 amino acid residues attributed to the "leader" of human GM-CSF. There follow codons specifying an alanine residue, a proline residue and a leucine residue. The proline and leucine residues duplicate the amino acids present at positions +2 and +3 of hpG-CSF, while the alanine residue is duplicative of the initial amino terminal (+1) residue of GM-CSF rather than hpG-CSF. Replacement of threonine by alanine was designed to be facilitative of proper host cell "processing off" of the GM-CSF leader by cellular mechanisms ordinarily involved in GM-CSF secretory processing.

Please replace the paragraph beginning on page 53, line 15 with the following rewritten paragraph:

Plasmid pSVDM-19 was digested with KpnI and the site was blunt ended with Klenow enzyme. Thereafter the DNA was cut with HindIII. The resulting large fragment was combined and ligated with the HindIII/PvuII fragment shown in ~~Table VH~~ FIG. 2 (isolated from plasmid Ppo2 as the second largest fragment resulting from HindIII digestion and partial digestion with PvuII) to form plasmid pSV-Ppol. The manufactured GM-CSF leader sequence fragment of ~~Table VH~~ FIG. 3 was then ligated into pSV-Ppol (following its cleavage with HindIII and Apal) to yield plasmid pSVGM-Ppol.

Please replace the paragraph beginning on page 54, line 17 with the following rewritten paragraph:

Finally, the genomic sequence whose isolation is described in Example 5 was employed to form an expression vector for mammalian cell expression of hpG-CSF. More specifically, pSVDM-19 was digested with KpnI and HindIII and the large fragment used in a four-way ligation with a synthetic linker with HindIII and NcoI sticky ends, as shown in ~~Table XXI~~ Table XIV. An NcoI-BamHI fragment containing exon 1 isolated from pBR322 (8500 hpG-CSF), a genomic subclone, and a BamHI-KpnI fragment containing exons 2-5 isolated from the plasmid pBR322 (8500 hpG-CSF genomic subclone). The resulting mammalian expression vector, pSV/ghG-CSF produced 1 to 2.5 $\mu\text{g/ml}$ of hpG-CSF from transformed COS cells.

Page 54, line 31, please replace "Table XXI" with --Table XIV--.

Please replace the paragraph beginning on page 55, line 8 with the following rewritten paragraph:

Recombinant hpG-CSF products of E. coli expression as in Example 7 had an apparent molecular weight of 18.8 kD when determined in reducing SDS-PAGE (as would be predicted from the deduced amino acid analysis of ~~Table VH~~ FIG. 2), whereas natural isolates purified as described in Example 1 had an apparent molecular weight of 19.6 kD. The presence of N-glycans associated with the natural isolates could effectively be ruled out on the basis of the lack of asparagine residues in the primary sequence of hpG-CSF in ~~Table VH~~ FIG. 2 and therefore a procedure was devised to determine if O-glycans were responsible for molecular weight differences between natural isolates and the non-glycosylated recombinant products. Approximately 5 µg of the natural isolate material was treated with neuraminidase (Calbiochem, LaJolla, California), a 0.5 µg sample was removed, and the remaining material was incubated with 4 mU O-Glycanase (endo-x-n-acetylgalactoseaminidase, Genzyme, Boston, Massachusetts) at 37°C. Aliquots were removed after ½, 2 and 4 hours of incubation. These samples were subjected to SDS-PAGE side by side with the E. coli derived recombinant material. After neuraminidase treatment, the apparent molecular weight of the isolate shifted from 19.6 kD to 19.2 kD, suggestive of removal of a ~~sialic~~ sialic acid residue. After 2 hours of treatment with O-glycanase, the molecular weight shifted to 18.8 kD -- identical to the apparent molecular weight of the E. coli derived material. The sensitivity of the carbohydrate structure to neuraminidase and O-glycanase suggests the following structure for the carbohydrate component: N-acetylneuraminic acid-α(2-6)(galactose acid-α(2-6)galactose β (1-3) N-acetylgalactosamine-R, wherein R is serine or threonine.

Please replace the paragraph beginning on page 57, line 18 with the following rewritten paragraph:

Natural isolates of pluripotent human G-CSF (hpG-CSF) and the recombinant pluripotent human G-CSF (rhG-CSF) were found to cause human bone marrow cells to proliferate and differentiate. These activities were measured in CFU-GM [Broxmeyer, et al., Exp. Hematol., 5, 87, (1971)] BFU-E and CFU-GEMM assays [Lu, et al., Blood, 61, 250 (1983)] using low density, non-adherent bone marrow cells from healthy volunteers. A comparison of CFU-GM, BFU-E and CFU-GEMM biological activities using either 500 units of hpG-CSF or rhG-CSF are shown in ~~Table XXII~~ Table XV below.

Page 58, line 1, please replace "Table XXII" with --Table XV--.

Please replace the paragraph beginning on page 58, line 34 with the following rewritten paragraph:

Colonies formed in the CFU-GM assay were all found to be chloracetate esterase positive and non-specific esterase (alpha-naphthyl acetate esterase) negative, consistent with the colonies being granulocyte in type. Both natural hpG-CSF and rhpG-CSF were found to have a specific activity of approximately 1×10^8 U/mg pure protein, when assayed by serial dilution in a CFU-GM assay. The BFU-E and CFU-GEMM data in ~~Table XXH~~ Table XV are representative of three separate experiments and similar to the data reported previously for natural hpG-CSF. It is important to note that the rhpG-CSF is extremely pure and free of other potential mammalian growth factors by virtue of its production in E.coli. Thus rhpG-CSF is capable of supporting mixed colony formation (CFU-GEMM) and BFU-E when added in the presence of recombinant erythropoietin.

Please replace the paragraph beginning on page 59, line 16 with the following rewritten paragraph:

It was previously reported that WEHI-3B(D⁺) cells and human leukemic cells from newly diagnosed leukemias will bind ¹²⁵I-labeled murine G-CSF and that this binding can be ~~complete~~ competed for by addition of unlabeled G-CSF or human CSF- β . The ability of natural hpG-CSF and rhpG-CSF to compete for binding of ¹²⁵I-hpG-CSF to human and murine leukemic cells was tested. Highly purified natural hpG-CSF (>95% pure; 1 μ g) was iodinated [Tejedor, et al., Anal. Biochem., 127, 143 (1982)] was separated from reactants by gel filtration and ion exchange chromatography. The specific activity of the natural ¹²⁵I-hpG-CSF was approximately μ Ci/ μ g 100 protein. Murine WEHI-3B(D⁺) and two human peripheral blood myeloid leukemic cell preparations (ANLL, one classified as M4, the other as M5B) were tested for their ability to bind ¹²⁵I-hpG-CSF.

Please replace the paragraph beginning on page 60, line 10 with the following rewritten paragraph:

Specific binding (cpm) was determined as total binding in the absence of a competitor (mean of duplicates) minus binding (cpm) in the presence of 100-fold excess of unlabeled hpG-CSF (non-specific binding). The non-specific binding was maximally 2503 cpm for WEHI-3B(D⁺) cells, 1072 cpm for ANLL (M4) cells and 1125 cpm for ANLL (M5B) cells. Experiments one and two were run on separate days using the same preparation of ¹²⁵I-hpG-CSF and ~~display~~ displayed internal consistency in the percent inhibition noted for 2000 units of hpG-CSF. Data obtained are reported in ~~Table XXH~~ below FIG. 9.

Please delete the entire text of page 61 (i.e., Table XXIII).

Please replace the paragraph beginning on page 62, line 1 with the following rewritten paragraph:

As shown in ~~Table XXIII~~ below FIG. 9, ^{125}I -hpG-CSF demonstrated binding to the WEHI-3B(D⁺) leukemic cells. The binding was inhibited in a dose dependent manner by unlabeled natural hpG-CSF or rhpG-CSF, but not by GM-CSF. In addition, binding of natural hpG-CSF to human myelomonocytic leukemic cells (ANLL, M4) was observed. The binding to these cells is paralleled in response to natural hpG-CSF in liquid cultures by differentiation into mature macrophages as judged by morphology. The absence of binding of natural ^{125}I -hpG-CSF to monocytic leukemic cells from another patient (ANLL, M5B) suggests that certain leukemias may differentially express or lack receptors for hpG-CSF. The ability of rhpG-CSF to compete for the binding of natural ^{125}I -hpG-CSF, similar to natural hpG-CSF, suggests that the receptors recognize both forms equally well.

Please replace the paragraph beginning on page 62, line 18 with the following rewritten paragraph:

These studies demonstrating the binding of natural ^{125}I -labeled hpG-CSF to leukemic cells are paralleled in culture by the ability of natural hpG-CSF to induce granulocytic and monocytic differentiation of light density bone marrow cells obtained from one patient with an acute promyelocytic leukemia (M3) and a second patient with an acute myeloblastic leukemia (M2). Cells from each patient were cultured for four days in medium alone or in the presence of 1×10^5 units of rhpG-CSF. Cells from the M3 control cultures incubated in medium alone were still promyelocyte in type; while cells cultured in the presence of rhpG-CSF showed mature cells of the myeloid type including a metamyelocyte, giant band form and segmented neutrophils and monocyte. The actual differentials for this patient, on 100 cells evaluated for the control, 100% promyelocytes, and for the rhpG-CSF treated cells 22% blasts plus promyelocytes, 7% myelocytes, 35% metamyelocytes, 20% band forms plus segmented neutrophils, 14% monocytes and 2% macrophages. Of note is the fact that one of the polymorphonuclear granulocytes still contained a prominent auer rod, suggesting that at least this cell represented a differentiated cell belonging to the leukemic clone. Cells from the second patient with a myeloblastic leukemia (M2) were also cultured for four days in the presence ~~of~~ or absence of rhpG-CSF. Visual analysis of M2 cells cultured in medium alone revealed large "blast-like" cells, some of which had nucleoli. Some of the M2 cells, when treated with rhpG-CSF, differentiated to mature segmented neutrophils displaying residual auer rods in the center neutrophil suggesting differentiation occurring in a cell belonging to the leukemic clone. The actual differentiation of 100 cells evaluated

morphologically revealed that control cells consisted of 100% blasts. The rhpG-CSF treated cells consisted of 43% blasts, 1% myelocytes, 15% metamyelocytes, 28% band forms plus segmented neutrophils, 2% promonocytes and 11% monocytes. The leukemic cells were also examined for differentiation at four other concentrations of rhpG-CSF (5×10^3 , 1×10^4 , 2.5×10^4 U/ml, data not shown). Even at the lowest concentration of rhpG-CSF tested (5×10^3 U/ml), there was significant differentiation (cells differentiated beyond myelocytes) of the M3 (50%) and M2 (37%) leukemic cells.

Please replace the paragraph beginning on page 65, line 7 with the following rewritten paragraph:

[Ser¹⁷]hpG-CSF, [Ser³⁶]hpG-CSF, [Ser⁴²]hpG-CSF, [Ser⁶⁴]hpG-CSF, and [Ser⁷⁴]hpG-CSF products prepared according to Example 9 were ~~assay~~ assayed for hpG-CSF activity in the ³H-thymidine uptake, CFU-GM, and WEHI3B D⁺ assays. In each assay, the [Ser¹⁷] analog had activity comparable to that of recombinant molecules having the native structure. The remaining analogs had on the order of 100-fold lesser activity in the ³H-thymidine uptake assay, 250-fold lesser activity in the CFU-GM assay, and 500-fold lesser activity in the WEHI-3B D⁺ assay. This data is supportive of the proposition that cysteines at positions 36, 42, 64 and 74 may be needed for full biological activity.

Please replace the paragraph beginning on page 66, line 3 with the following rewritten paragraph:

In addition to naturally-occurring allelic forms of hpG-CSF, the present invention also embraces other hpG-CSF products such as polypeptide analogs of hpG-CSF and fragments of hpG-CSF. Following the procedures of the above-noted published application by Alton, et al.(WO/83/04053) one may readily design and manufacture genes coding for microbial expression of polypeptides having primary conformations which differ from that herein specified for in terms of the identity or location of one or more residues (e.g., substitutions, terminal and intermediate additions and deletions). Alternately, modifications of cDNA and genomic genes may be readily accomplished by well-known site-directed mutagenesis techniques and employed to generate analogs and derivatives ~~of~~. Such products would share at least one of the biological properties of hpG-CSF but may differ in others. As examples, projected products of the invention include those which are foreshortened by e.g., deletions; or those which are more stable to hydrolysis (and, therefore, may have more pronounced or longer lasting effects than naturally-occurring); or which have been altered to delete one or more a potential sites for o-glycosylation (which may result in higher activities for yeast-produced products); or which have one or more cysteine residues deleted or replaced by, e.g., alanine or serine residues and are potentially

more easily isolated in active form from [microbial] microbial systems; or which have or more tyrosine residues replaced by phenylalanine and may bind more or less readily to hpG-CSF receptors on target cells. Also comprehended are polypeptide fragments duplicating only a part of the continuous amino acid sequence or secondary conformations within hpG-CSF, which fragments may possess one activity of (e.g., receptor binding) and not others (e.g., colony growth stimulating activity). It is noteworthy that activity is not necessary for any one or more of the products of the invention to have therapeutic utility [see, Weiland, et al., Blut, 44, 173-175 (1982)] or utility in other contexts, such as in assays of hpG-CSF antagonism. Competitive antagonists may be quite useful in, for example, cases of overproduction of hpG-CSF.